NEW CARDENOLIDES FROM THE SEEDS OF CORONILLA HYRCANA

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Coronilla hyrcana Prilipko, family Leguminosae, is an endemic species growing in Azerbaijan [1]. The paper chromatography of an extract of the seeds (Figs. 1 and 2, samples 1 and 6) showed the presence of seven substances of cardenolide nature, of which two have been isolated – hyrcanoside and deglucohyrcanoside (Fig. 1, samples 2 and 3).

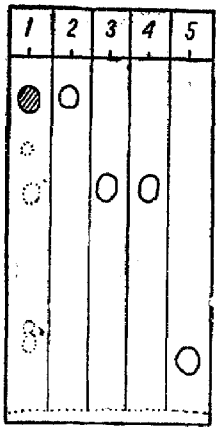


Fig. 1. Toluene – butan-1-ol (3:1) – water (35%) system, 4 hr, 18° C. 1) Total cardenolides of the seeds of Coronilla hyrcana; 2) hyrcanoside (I); 3) deglucohyrcanoside (VII); 4) hyrcanoside (I) after 13-15 min enzymatic hydrolysis; 5) hyrcanoside (I) after enzymatic hydrolysis for 24 hr.

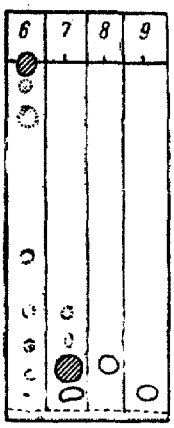
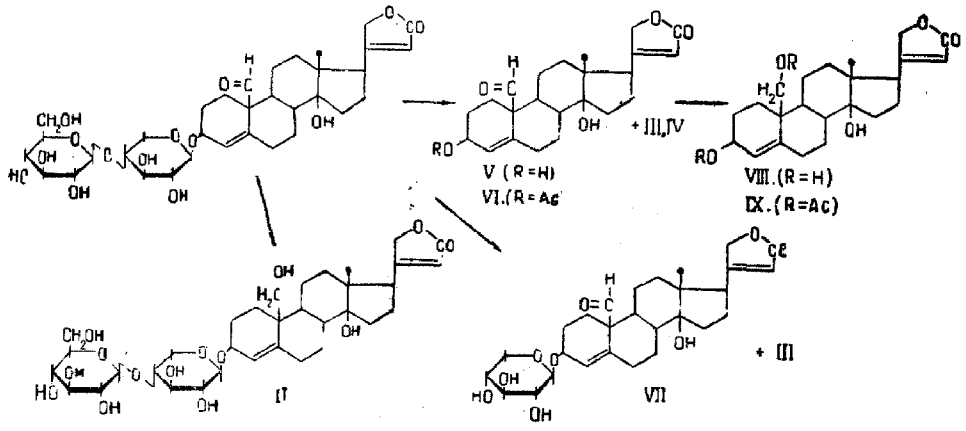


Fig. 2. Chloroform – tetrahydrofuran – formamide (50:50:6.5) system, 3 hr, 18° C. 6) Total cardenolides of the seeds of Coronilla hyrcana; 7) total cardenolides after enzymatic hydrolysis; 8) hyrcanogenin (V); 9) substance E.

After enzyme treatment of the total glycosides (cf. Fig. 2, sample 7), another two substances were isolated – hyrcanogenin and substance E (cf. Fig. 2, samples 8 and 9).

The first substance – hyrcanoside (I) with the empirical formula $C_{34}H_{48}O_{14}$ – is readily reduced by sodium borohydride to hyrcanosidol (II). The acid hydrolysis of (I) by the method of Mannich and Siewert [2] led to the liberation of D-xylose and D-glucose, the aglycone undergoing far-reaching degradative changes. To obtain the unchanged aglycone (V) we made use of the properties of D-glucosides and D-xylosides of undergoing hydrolysis by the enzymes of the fungus Aspergillus oryzae [3, 4]. The aglycone of hyrcanogenin (V) isolated after the enzymatic cleavage of hyrcanoside gave a monoacetyl derivative (VI) on acetylation. The genin (V) was reduced by $NaBH_4$ to hyrcanogenol (VIII). The latter gave a diacetate (IX) on acetylation.



In the UV region, hyrcanogenin has two absorption maxima: the first at 218 mμ (log ε 4.17), which is characteristic for a butenolide ring, and the second at 307 mμ (log ε 2.11), which corresponds to an aldehyde group at C_{10}. The presence of an aldehyde group in the aglycone (V) is also confirmed by the results of the oxidation of its acetate (IV) with chromium trioxide in glacial acetic acid [5]. The yield of acid product is comparatively low as is found in the oxidation of pachygenin [6].

The investigations carried out have enabled us to establish that the aglycone (V) contains two hydroxy groups. One of them undergoes acetylation and is located at C_3 and the other (at C_{14}) does not undergo acetylation and is tertiary. The results of elementary analysis and the UV spectra give grounds for assuming that the molecule of hyrcanogenin has a double bond. This is shown by the high intensity of the absorption of the aldehyde group (λ_{max} 307 mμ; log ε 2.11). Some authors [6–8] explain such a phenomenon by the homoconjugation of the aldehyde group and the double bond.

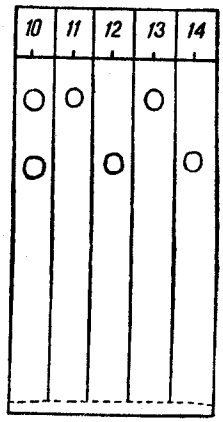


Fig. 3. Butan-1-ol–acetic acid–water (4:1:5) system, 17 hr, 18° C. (10) Sugar moiety of hyrcanoside (I) after acid and enzymatic hydrolysis; 11) sugar moiety of hyrcanoside (I) after stepwise enzymatic hydrolysis; 12) sugar moiety of deglucohyrcanoside (VII) after enzymatic hydrolysis; 13) D-glucose; 14) D-xylose.

On analyzing the influence on the molecular rotation of the introduction of a double bond into various positions of the steroid nucleus [9] we came to the conclusion that the double bond in hyrcanogenin (V) is most probably in the 4,5-position. The negative reaction of substance (V) and its acetyl derivative (VI) with tetranitromethane also indicates the presence of an allyl grouping in ring A, i.e., a 4-en-3-ol [10, 11].

To confirm this position, we compared the increments of the molecular rotations for a double bond of the aglycone under investigation (V) and its reduced form (VIII) with the increments for Δ^4 in scilliglaucosidin and 4-anhydrostrophanthidol. The results obtained confirm the proposed position of the double bond. Furthermore, a convincing proof of the structure of hyrcanogenin (V) is the identity of the properties of hyrcanogenol (VIII) and 3β, 14β, 19-trihydroxycard-4, 20(22)-dienolide [12].

In elucidating the positions of attachment of the sugar residues in hyrcanoside (I) we carried out a stepwise hydrolysis (cf. Fig. 1, sample 4, and Fig. 3, sample 11), as a result of which we isolated a monoside – deglucohyrcanoside (VII) – and D-glucose (III). Further enzymatic hydrolysis of (VII) gave the aglycone hyrcanogenin (V) and D-xylose (IV) (cf. Fig. 3, sample 12).

The configuration of the glycosidic centers was determined by Klyne's method [13] and it was found that both sugars have a β-glycosidic bond. Their stability to acid hydrolysis [14, 15] shows that both the D-glucose and the D-xylose in the glycoside are present in the pyranose form.

A comparison carried out of the positions of attachment of the sugar residues in the carbohydrate chain of various glycosides of the cardenolide series [16] gives grounds for assuming that in substance (I) the glucose is linked with the xylose by a 1 → 4 bond.

Thus, hyrcanoside (I) is 3-β-(O-β-D-xylopyranosyl- < 4 ← 1 >-O-β-D-glucopyranosyl)-14β-hydroxy-19-oxocard-4, 20(22)-dienolide.

The second substance – deglucohyrcanoside –, obtained after separating the total cardenolide products of enzymatic hydrolysis by their physicochemical properties, was identical by its coloration with 84% sulfuric acid, a mixed melting point test, and paper chromatography (cf. Fig. 1, samples 3 and 4) with the monoside (VII) isolated after the enzymatic cleavage of hyrcanoside (I) and is 3β-(O-β-D-xylopyranosyl)-14β-hydroxy-19-oxocard-4, 20(22)-dienolide.

The third substance proved to be identical with hyrcanogenin (V), the structure of which can be characterized as 3β, 14β-dihydroxy-19-oxocard-4, 20(22)-dienolide.

Some physicochemical properties of the fourth substance, provisionally known as substance E have been determined.

Experimental

The adsorption chromatography was carried out on neutral alumina (activity grade III). For analysis the substances were dried in vacuum (10^{-2} mm Hg) at 110°–115° C for 4–5 hr over P_2O_5. The melting points were determined on a Kofler instrument.

Isolation of the cardenolides. The cardenolides were extracted from 5.1 kg of the ground seeds of Coronilla

hyrcana, defatted with petroleum ether, with 50 *l* of 70% ethyl alcohol. The extract was evaporated in vacuum to 3.5 *l*, and then the solvent was evaporated off to dryness and the residue was dissolved in 1 *l* of water and filtered through a column of alumina (10 × 8 cm) with subsequent washing with water until all the cardenolides had been eluted (3.7 *l*). The eluate was divided into two parts: 2.8 and 0.9 *l*.

To separate the cardenolide mixture into individual fractions, 2.8 *l* of the aqueous eluate was treated with chloroform and then with mixtures of chloroform and alcohol (8.5:1.5) and (2:1).

The 8.5:1.5 chloroform-alcoholic extract gave 0.12 g of the deglucohyrcanoside and the 2:1 extract gave 6.1 g of hyrcanoside.

Enzymatic degradation of the total glycosides. Three g of an enzyme preparation from the fungus Aspergillus oryzae in 15 ml of water (pH of the medium 5.0–5.5) was added to 0.9 *l* of the eluate containing the mixture of glycosides and the mixture was left for a day at 45°–47° C. After the completion of fermentation, the liquid was treated with a mixture of chloroform and 15% of alcohol, and the extract was evaporated to dryness (residue 2.79 g).

The mixture of cardenolides was separated on a column of alumina (10 × 3 cm). Elution was carried out with benzene containing 10–70% of chloroform and then with chloroform containing various amounts of alcohol.

The 9:1 and 8:2 benzene-chloroform fractions gave 0.11 g of substance E and hyrcanogenin was isolated from the chloroform fraction.

Hyrcanoside (I). This substance has mp 200°–210° C (from methanol); $[\alpha]_D^{20}$ +7.4° (c 0.94; methanol). The color reaction with 84% sulfuric acid gives a coloration changing with time: 0 min – green with an instantaneous transition to brown-lilac; 1–20 min – brown-lilac; 20–40 min – dark lilac; 40–90 min – dirty purple. For the paper chromatogram of substance (I), see Fig. 1, sample 2.

Found, %: C 59.90; H 7.16; mol. wt. 682.78 (lactone titration). Calculated for $C_{34}H_{48}O_{14}$, %: C 59.99; H 7.11; mol. wt. 680.72.

Reduction of (I) to hyrcanosidol (II). A solution of 1.27 g of hyrcanoside (I) in 50 ml of methanol was cooled to +3° C, or +5° C, treated with 30 mg of sodium borohydride, and left in the cold for 4 hr. Then the methanol was distilled off, the residue was dissolved in 15 ml of water, and the glycoside was extracted with a mixture of chloroform and alcohol (2:1). The extract was evaporated and the residue was purified on a column of silica gel (8 × 1.5 cm) with subsequent elution of the glycosides with a mixture of chloroform and alcohol (1:1). After the evaporation of the eluate followed by crystallization of the residue from moist isopropyl alcohol, 0.97 g of small nodules with mp 163°–167° C, $[\alpha]_D^{19}$ −26.6° (c 0.94; methanol) was obtained. With 84% sulfuric acid, hyrcanosidol gives a crimson coloration.

Found, %: C 59.74; H 7.32; mol. wt. 673.0 (lactone titration). Calculated for $C_{34}H_{50}O_{14}$, % : C 59.81; H 7.38; mol. wt. 682.74.

Acid hydrolysis of (I). A suspension of 50 mg of hyrcanoside in 50 ml of acetone was treated with 0.5 ml of conc. hydrochloric acid. The progress of hydrolysis was followed by means of paper chromatography in the chloroform–formamide system and others. After nine days, only traces of the starting material remained together with a number of substances of cardenolide nature with low polarities. The further treatment was carried out by the usual method [17].

The aqueous residue after the elimination of chloride ion with silver carbonate was found by paper chromatography to contain D-glucose and D-xylose (cf. Fig. 3, sample 10). The chloroform extracts were not investigated.

Enzymatic hydrolysis of (I). A solution of 1.0 g of substance (I) in 50 ml of water was treated with 1.0 g of an enzyme preparation from the fungus Aspergillus oryzae in 20 ml of water (pH of the medium 5.0–5.5) and the mixture was kept at 47° C. After a day, one spot was found on the chromatogram (see Fig. 1, sample 5). The fermentation product was extracted with chloroform (3 × 50 ml). The residue after the chloroform had been distilled off was dissolved in a small amount of 96% ethyl alcohol, the solution was filtered through a layer of alumina (2 × 0.5 cm), and the filtrate was evaporated to dryness. This yielded 315 mg of hyrcanogenin (V) with mp 227°–235° C (from methanol and water); $[\alpha]_D^{19}$ + 94° [c 1.32; chloroform–alcohol (3:2)].

The color reaction with 84% sulfuric acid gave a coloration changing with time: 0 min – bright green; 1–90 min – – brownish purple. The reaction with tetranitromethane was negative.

Found, %: C 71.38; H 7.78; mol. wt. 386.09 (lactone titration). Calculated for $C_{23}H_{30}O_5$, %: C 71.48; H 7.82; mol. wt. 386.5.

Acetyl derivative of hyrcanogenin (VI). A solution of 124 mg of the aglycone (V) in 3 ml of absolute pyridine was treated with 3 ml of acetic anhydride. The reaction mixture was left for a day, after which it was poured into

300 ml of ice-water. This gave 105 mg of substance (VI) with mp 189°–197° C (from acetone-ether); $[\alpha]_D^{20}$ + 53.2° (c 1.41; chloroform).

Found, %: C 73.11; H 7.59; mol. wt. 431.0. Calculated for $C_{25}H_{32}O_6$, %: C 73.03; H 7.52; mol. wt. 428.5.

Oxidation of (VI). A solution of 19 mg of the aglycone acetate (VI) in 1.0 ml of glacial acetic acid was treated with 0.1 ml of a solution of chromium trioxide in glacial acetic acid. After the mixture had stood for an hour, it was worked up as described previously [18].

This gave about 35% of an acid fraction of the aglycone acetate. The amorphous substance isolated had R_f 0.38 on paper chromatography in the benzene–formamide system and possessed a considerably greater polarity than the starting material (R_f 0.75) as has been observed for several cardenolides oxidized at C_{19} [19].

Reduction of hyrcanogenin (V) to hyrcanogenol (VIII). Under conditions similar to those for hyrcanoside, 124 mg of the aglycone (V) in 15 ml of methanol was reduced with 25 ml of sodium borohydride [11]. The residue after distilling off the methanol was dissolved in 2 ml of ethanol and 25 ml of water was added. The reduced aglycone was extracted with chloroform containing 2% ethanol, evaporated to 5 ml and, for purification, filtered through a layer of alumina (1.5 × 0.5 cm) with subsequent elution with a mixture of chloroform and alcohol (9:1). The filtrate was evaporated and the residue was crystallized from methanol–ether. This gave 108 mg of crystals with mp 238°–246° C; $[\alpha]_D^{20}$ + + 27.4° (c 0.51; chloroform–methanol (8:2)]. The color reaction with 84% sulfuric acid gave a coloration changing with time: 0 min — orange, changing instantaneously to red; 1–90 min — crimson-cherry red. On paper chromatography in the chloroform–formamide system, R_f 0.12.

Found, %: C 71.52; H 8.41; mol. wt. 387.76. Calculated for $C_{23}H_{32}O_5$, %: C 71.64; H 8.36; mol. wt. 388.5.

Acetyl derivative of hyrcanogenol (IX). A 60 mg sample of hyrcanogenol (VIII) was acetylated as described for hyrcanogenin (V). The acetyl derivative was crystallized from ether with the addition of a few drops of methanol. The weight of crystals was 48 mg, mp 177°–183° C; $[\alpha]_D^{19}$ +23° (c 0.78; chloroform).

Found, %: C 68.70; H 7.73; mol. wt. 476.4. Calculated for $C_{27}H_{36}O_7$, %: C 68.83; H 7.68; mol. wt. 472.5.

A determination [20] showed the presence of two acetyl residues. Oxidation of the acetyl derivative (IX) under the same conditions as for the acetate of hyrcanogenin (VI) led to no changes in the diacetyl derivative of hyrcanogenol.

The sugar residue from (I) after enzymatic hydrolysis. The aqueous residue after the extraction of the hyrcanogenin (V) was evaporated to 20 ml and was treated with 80 ml of boiling 96% ethyl alcohol to eliminate the enzyme preparation. The precipitate was filtered off and washed with 80% ethanol. The filtrate was again evaporated and the operation was repeated three times. The aqueous residue purified in this way was investigated for the presence of sugars liberated by the enzyme preparation (see Fig. 3, sample 10). D-Xylose and D-glucose were found.

Stepwise hydrolysis of (I). The method for this was proposed by P. I. Gvozdyak. A solution of 1.0 g of substance (I) in 20 ml of water was treated with 1.0 g of an enzyme preparation from the fungus Aspergillus oryzae, dissolved in 10 ml of water, and the mixture was kept at 45°–47°C for 13–15 min. After this period there was none of the starting material in the reaction mixture (see Fig. 1, sample 4). The subsequent treatment was carried out as described previously [3]. This gave 478 mg of acicular crystals of substance (VIII) with mp 197°–200° C (from methanol), $[\alpha]_D^{19}$ +24.46° [c 0.94; methanol–pyridine (8:2)]. The reaction with 84% sulfuric acid formed colorations changing with time similar to those of substance (I). For a paper chromatogram of substance (VIII), see Fig. 1, sample 4.

Found, %: C 65.02; H 7.32; mol. wt. 516.1 (lactone titration). Calculated for $C_{28}H_{38}O_9$, %: C 64.84; H 7.39; mol. wt. 518.58.

Sugar residue (III) after the degradation of (I) to the monoside (VII). The aqueous residue after the extraction of substance VII with a mixture of chloroform and alcohol (8:2) was treated as described above.

From 0.32 g of purified sirup was crystallized 0.037 g of a substance with mp 135°–137° C (from ethanol). Chromatography on paper (see Fig. 3, sample 11) and also a mixed melting point test showed that the sugar isolated (III) was identical with D-glucose.

Sugar residue of the monoside (VII). The enzymatic cleavage of substance (VII) was carried out under the same conditions as for (I). This gave the aglycone (V) and a sugar (IV) with mp 143°–145° C (from ethanol with a trace of ether).

The results of paper chromatography (cf. Fig. 3, samples 12 and 14) and a mixed melting point test showed the identity of the sugar component of (VII) with D-xylose (IV).

Deglucohyrcanoside (VII). This was isolated from the chloroform–alcohol fraction (8.5:1.5). It crystallized

from methanol in the form of colorless crystals with mp 197°–200° C; $[\alpha]_D^{19}$ + 25° [c 1.0; methanol–pyridine (8:2)]. The reaction with 84% sulfuric acid formed colorations similar to those of (I) and (VII). For a paper chromatogram of substance (VII) see Fig. 1, sample 3.

Found, %: C 64.98; H 7.34; mol. wt. 512.2 (lactone titration). Calculated for $C_{28}H_{38}O_9$, %: C 64.84; H 7.39; mol. wt. 518.58.

After enzymatic hydrolysis the aglycone hyrcanogenin (V) and D-xylose (IV) were isolated. A mixture with the deglucohyrcanoside (VII) obtained by the enzymatic cleavage of (I) gave no depression of the melting point.

Hyrcanogenin (V). This was isolated after the enzymatic hydrolysis of the total glycosides of the seeds with mp 226°–234° C, $[\alpha]_D^{19}$ +91° [c 0.88; chloroform–alcohol (92:8)]. Its color reaction with 84% sulfuric acid, its physicochemical properties, a mixed melting point test, and the results of paper chromatography in various systems showed the authenticity of the substance under investigation as hyrcanogenin (V), the aglycone of the glycosides (I) and (VII).

Substance E. This was crystallized from methanol and ether, mp 185°–190° C, $[\alpha]_D^{20}$ –60° [c 0.93; chloroform–alcohol (92:8)]. The reaction with 84% sulfuric acid forms a coloration changing with time: 0 min – green, changing to dark brown; 1 min – brown; 2–90 min – dirty green. On paper chromatography in the benzene–formamide system, R_f 0.54.

Summary

Four substances have been isolated from the seeds of Coronilla hyrcana: hyrcanoside, deglucohyrcanoside, hyrcanogenin, and substance E.

It has been established that hyrcanoside (I) has the structure 3β-(O-β-D-xylopyranosyl-<4←1>-O-β-D-glycopyranosyl)-14β-hydroxy-19-oxocard-4, 20(22)-dienolide; deglucohyrcanoside (VII) is 3β-(O-β-D-xylopyranosyl)-14β-hydroxy-19-oxocard-4, 20(22)-dienolide; and hyrcanogenin (V) is 3β, 14β-dihydroxy-19-oxocard-4, 20(22)-dienolide. Both the aglycone hyrcanogenin (V) and its glycosides (I) and (VII) are new compounds.

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